ASPB Pioneer Member

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In my career and life, I have been fortunate to experience serendipity – the good luck in making unexpected and fortunate discoveries. However, when I made a choice that wasn't good for me, a little voice said, quietly and then louder, you must make a change or do something over. When I followed this instruction, the voice became silent and once again serendipity could come again. In this autobiography, I describe how this process occurred during my career, and as you will see, a scientific career can be an up and down journey.

How my career started.

I have had mentors throughout my life. My first mentors were my father, Albert, and my mother, Lillian. They taught me to work hard and follow my dreams. In my house it was a duty to educate oneself. My parents promised I could go to any public college of my choice if I was accepted. However, sitting around the dinner table after I was accepted by UC San Diego, my parents suggested I stay at home and spend two years at a nearby State College. I looked at them and said three words, "but you promised." They remembered and there was no more discussion; I went to UC San Diego. They were honorable mentors, and they taught me to be an honorable mentor during my career.

My freshman year (1969) at UC San Diego, Revelle College, was difficult. We were required to study calculus, physics, chemistry, and humanities. I was not sufficiently focused to keep up with the best students and teachers. Even though I received C grades (average), I knew I could not build on my current state of knowledge. I was saved by chance when I met Ann, my future wife, at UC San Diego. She supported me retaking some science courses. She also told me to NEVER skip a class, even if I was not prepared. I followed her instructions.

In those days, there were very few handouts, no laptops, no Word files or PowerPoint, etc. The professor lectured and I scribbled notes in class. For me, hearing the lecture and scribbling notes was not enough. I had to learn how I learn. So, after the class, as soon as possible, I rewrote my notes in clear sentences and drew figures in a fresh notebook. If I reached the point where I couldn't explain something, it meant I needed help! This approach made it possible for me to understand new research concepts and, later, teach classes clearly throughout my career.

At the end of my sophomore year, I had to choose a major for a B.A. degree. I loved everything, math, biology, physics, and literature. But Ann was practical. Her major was biology, and she encouraged me to declare the same major, because it could lead to many different productive careers. Also, the war in Viet Nam was in progress, and there was a belief that certain aspects of science were feeding technology supporting it. Biology, however, was all about medicine and agriculture, how organisms grow, develop, and evolve. So, I chose biology without knowing precisely in which direction I would go.

In my junior and senior years, I was ravenous to learn, and I took extra math classes and the organic and physical chemistry courses designed for math and chemistry majors. A genetics lab course, taught by Professor Dan Lindsley, really impacted my career. I was given wild-type and mutant *Drosophila* lines: the goal was to identify mutant phenotypes, make a hypothesis, do crosses, examine the progeny, and make a genetic conclusion (dominant vs recessive; linked or unlinked). After each cross I had to meet with the teaching assistant and defend my hypothesis. This created my foundation for understanding and teaching biological research, and it led me to doing biochemical and genetic research.

In 1973, I became a graduate student in the Department of Molecular Biology at UC Berkeley. My mentor was a brilliant scientist, Professor Harrison Echols. He taught me how to do experiments using positive and negative controls, which are needed to make justifiable conclusions. I was fortunate to do research using a model organism (easy to propagate and amenable to genetic and biochemical experiments) called bacteriophage *Lambda*, a virus that infects *E. coli*. *Lambda* has a small

genome with a map of 50 genes and a variety of mutations that display phenotypes. *Lambda's* life cycle has a sophisticated developmental switch. After infection, *Lambda* can follow a viral reproduction and lytic path, by replicating its genome, synthesizing capsids, and releasing 50 progeny phage in 45 minutes. Alternatively, it can follow a lysogenic path, where it inserts its DNA into the *E. coli* genome, which represses most of *Lambda's* genes and makes the bacterium immune to further *Lambda* infection.

One would think such a short life span (45 minutes) would allow me to make rapid progress, and Professor Echols always had confidence that I would. However, five years passed without obtaining publishable data. Sitting at my desk, staring at the wall, I concluded I was responsible, not my mentor, for my success in the lab. I must depend on myself to design and execute my experiments. I was determined to break the publication wall even if I had to hit it with my head. This determination stayed with me throughout my career. Surprisingly, the first experiment I designed by myself produced publishable data. Ultimately, I graduated with five multi-author publications about the *Lambda* lysogenic pathway, describing how specific promoters transiently express genes for inserting its genome into the *E. coli* chromosome and repress the viral genes.

Stephen Chung, a postdoctoral fellow in the lab, was a valuable mentor for me. In those days there were no restriction enzymes nor the ability to sequence genomes, like that of *Lambda*. Chung taught me how to do genetic crosses that created lambda phage with unique genomes essential for our studies. We were able to visually identify the rare *Lambda* genome recombinants needed for our experiments. Later, when I was a professor in UC Berkeley in the 1990's, I used visual methods to identify rare floral reproductive mutations in a model plant, Arabidopsis.

It seemed natural to be a postdoctoral fellow in a laboratory studying eukaryotic viruses, and I was offered and accepted a position and stipend in a well-known UC San Francisco lab. However, as time went by, I heard that voice inside telling me this was not a good choice. Why? First, the study of viruses was a very competitive field. Second, this laboratory studied viruses affecting human health, and while I was concerned about my own health, I felt I would be at a disadvantage without an M.D. degree. I declined the position well before I was scheduled to arrive. So, I started over. I went to a reading room to look at postdoctoral fellow advertisements in *Science* and *Nature* magazines. In less than an hour I found the mentor that I wanted, Professor Robert (Bob) B. Goldberg, a distinguished plant biologist at UCLA. It seemed that plant biology was a burgeoning field, where there was much to learn, and technology was making it possible to do cutting edge experiments with plants. I felt I could breathe again! I was Bob Goldberg's postdoc from 1979 – 1983, and as I will describe below, he was a mentor my entire career.

Bob Goldberg was a teacher in the classroom as well as the lab. He taught me plant biology and how to do experiments with plants. He provided an understanding of plant genome structure based on nucleic acid hybridization kinetics, a method pioneered by his mentors, Professors Roy Britten, and Erik Davidson. Goldberg described the structure of eukaryotic plant genomes and how they are composed of multiple classes of repeated DNA sequences. Advances in molecular biology techniques, particularly DNA cloning and sequencing, gave us the ability to dissect plant genomes and analyze the small parts corresponding to single-copy genes, gene families, copies of different kinds of transposons, and the many small repeats that comprise structural DNAs. This knowledge made it possible for me to become a plant molecular biologist and be hired at UC Berkeley.

My earliest contribution to Goldberg's lab was knowledge and experience working with bacteriophage *Lambda*. It was hard to imagine that my time studying this bacteriophage would help me study the soybean genome. That's because *Lambda* became a vector for cloning DNA and making genomic libraries. We isolated soybean DNA, sheared it into 15 kilobase pieces, and ligated them into a modified *Lambda* vector called Charon 4 (Blattner et al 1977). With a cDNA corresponding to the glycinin seed storage protein mRNA, which encodes a nutritionally important protein for humans and livestock, we were able to isolate glycinin genes from a genomic library. I felt like I had landed on the moon! Everything we learned was fundamentally new. We determined there were apparently three glycinin genes in the soybean genome and the large accumulation of the glycinin mRNA in developing seeds was not due to amplification or

rearrangement of the genes during embryogenesis. We discovered glycinin genes contain introns that differ in number from those found in the adjacent genes expressed in leaves. The leaf genes encoded rare mRNAs, suggesting they had completely different regulatory mechanisms. At the time, these were breakthroughs in understanding plant genomes, and we realized that in many ways we were catching up with laboratories that studied animal genomes. I knew there was a bright future for plant molecular biology.

In 1983, it was time for me to find a real job. I was interviewed at two biotech companies and two universities. How could I choose? Initially, I chose a small plant biotech company in Sacramento that focused on using molecular biology to improve agricultural plants. I looked forward to doing work the company desired. I did not have to raise money for the research, and I would get a very good salary and stock options, etc. It took several months for that little voice to wake me up! I realized that for me to be happy, I must be responsible for my own research. Therefore, I had to be successful writing grants for the National Science Foundation and/or the U.S. Department of Agriculture. I had to put that fear behind me and take the chance I would get funded.

There was one problem, I had declined the offer of an Assistant Professorship at UC Berkeley. I called the department there and asked the chairman of the Search Committee whether the job was still open. He said unfortunately no, as the Dean had retracted the position. So, I thanked him and prepared to apply to other universities. But a week later someone in Goldberg's lab said, "Hey Bob, someone is calling you from UC Berkeley! He asked if you still want the job?" I said yes, and I started as an Assistant Professor in the Division of Plant Molecular Biology at UC Berkeley. Thirty-four years later, 2017, I retired from in the Department of Plant and Microbial biology at Berkeley. I couldn't have imagined a better place to work.

Initially, I set up my lab and studied how the ethylene hormone regulates tomato fruit ripening. Using molecular biology approaches, I identified genes that respond to ethylene, and, in collaboration with Professor Alan Bennett at UC Davis, we used tomato genetics and biochemistry to understand how the polygalacturonase enzyme influences tomato fruit softening. With the support of grants from the National Science Foundation and United States Department of Agriculture, we published nine manuscripts, a number sufficient for me to be promoted to a tenured Associate Professor position in 1989.

After receiving tenure, I continued to study tomato fruit ripening, but I was not satisfied with the project. Working with tomatoes was like driving a model-T Ford pickup truck. It takes almost nine months to obtain ripening fruit, and knowledge of tomato genetics was rather limited. In the rear-view mirror I saw a speeding sports car, a model plant called Arabidopsis, that had been developed by visionary scientists. I called Bob Goldberg and said, "When I think of my tomato research, I feel like I am walking down a hallway that is becoming more and more narrow! What should I do?" Bob said two things, "Drop it! I'm coming to see you; let's have lunch and talk."

That conversation was very fruitful. We went to the second Arabidopsis Conference with the idea of using it to learn more about seed development. We learned Arabidopsis is conveniently small with a short life span, just six weeks from seed to seed, which allowed us to do experiments much more quickly. We could grow thousands of plants in a small greenhouse and find mutants, or we could use the large collection of mutants available in The Arabidopsis Resource Center (TAIR). Arabidopsis has a small genome, only 10^8 base pairs, and was the first plant genome sequenced. Multiple methods were available to identify and clone a mutant allele and compare it with the wildtype allele. Importantly, Arabidopsis carries out all the normal functions of seed development.

We were ready to start, and Bob Goldberg had a grand plan. He created *The Seed Institute,* initially comprised of members of his laboratory, my lab, and those of John Harada (UC Davis) and Gary Drews (University of Utah), tenured professors who previously worked for Bob. Our goal was to collaborate on understanding how the Arabidopsis seed develops. We shared the work, funding, and authorship on published manuscripts. The *Seed Institute* had an annual three-day meeting every year, where all the labs presented their research. It was a great way to get feedback and create ideas for future experiments.

One of our first projects was working with Professor Ken Feldmann, who kindly let us search the mutant Arabidopsis lines he had created by

insertion of the T-DNA from *Agrobacterium*. We screened these lines twice and obtained many embryo lethal mutations. They were distinguished as heterozygous plants that produced 25% defective seeds. These lines were easily propagated, because 75% of the viable seeds represented 1/3 wild-type and 2/3 heterozygotes that were viable.

Arabidopsis reminded me of how I studied *Lambda* as a graduate student. My initial contribution to the Seed Institute was a focus on the female ovule side of embryogenesis, by looking for female sterile Arabidopsis lines. We found two distinct mutations, the genes of which corresponded to two transcription factor proteins: BELL1, which encodes a homeodomain protein involved in pattern formation in the Arabidopsis ovule primordium, and AINTEGUMENTA, which is related to the floral homeotic transcription factor, APETALA2, and controls ovule and female gametophyte development and organ size. The resulting publications were important when it came time to write grants on Arabidopsis rather than tomato fruit ripening. My lab had made the transition!

Bob Goldberg was one of the founders of a new agricultural biotechnology company, Ceres, that provided funds for seven years to support research by Seed Institute laboratories, which significantly increased the personnel in our labs. By the time this funding ended, my lab had made significant research advances that made it possible to obtain grants from the National Institute of Health and the National Science Foundation.

What was my laboratory's most impactful contribution to plant science research.

My most impactful contribution came from my fascination with the angiosperm female gametophyte, the progenitor of the embryo and endosperm. The *Arabidopsis* female gametophyte is formed within the ovule. A megaspore mother cell undergoes meiosis, and one haploid spore undergoes three mitotic divisions to form an 8-nucleus, 7-celled female gametophyte containing the egg, central, synergid, and antipodal cells. Before fertilization, a diploid nucleus is formed in the central cell by the fusion of two haploid nuclei.

In the anther, pollen mother cells undergo meiosis and produce haploid microspores, which undergo asymmetric mitosis, producing a large vegetative cell and a smaller generative cell. The generative cell, engulfed in the cytoplasm of the vegetative cell, undergoes a second mitosis to form two identical haploid sperm cells. After maturation, pollen is shed from the anther. Upon binding to the stigma, the trinucleate pollen grain rehydrates and produces a tube (cell wall) that grows through the ovule and transports two sperm cells to the female gametophyte, where fertilization of both the egg and central cell generates a diploid embryo and a triploid endosperm.

Mitosis in the embryo generates organs (axis and cotyledon), tissues (protoderm, procambium, and ground meristem), and meristems (shoot and root). Initially, nuclei in the fertilized central cell form a syncytium, which following its cellularization produces a multicellular endosperm. Some endosperm cells are repositories for storage proteins, lipids, and starch, while others mediate the transfer of these nutrients from maternal tissues. Ultimately these nutrients accumulate in the embryo cotyledons. Maternal cell layers surround and protect the developing embryo and endosperm, creating a seed coat derived from the ovule integuments. When viable reproduction occurs, the Arabidopsis silique (like a pea pod) elongates to make room for the developing seeds.

Our impactful research began with a simple idea and a question that came together at the same time. The idea came to me when I visited Professor Daphne Pruess' laboratory ,where I had learned about Arabidopsis conditional male sterile plants. The question came to me a day later when my graduate student, Leonore Reiser, asked this question, "Can we transform sexually reproducing *Arabidopsis* to an asexual reproducing plant?"

Plant asexual reproduction (apomixis) occurs when there is no genetic contribution from pollen. Apomitic plants occur in ~2% of angiosperm genera, with different species using different mechanisms. In plants with diplospory apomixis, the megaspore mother cell does not undergo meiosis: one diploid cell undergoes three mitotic divisions to form an 8-nucleus, 7-cell female gametophyte with diploid cells, whereas a tetraploid nucleus is formed in the central cell by the fusion of two diploid nuclei. Without fertilization, the tetraploid central cell generates the endosperm, the diploid egg undergoes embryogenesis, and the offspring and the mother plant are genetically identical. Apomitic plants could be

agriculturally valuable for making hybrid seed.

Our goal was to visually identify a rare mutation in *Arabidopsis* that would display aspects of apomixis – embryogenesis, endosperm, seed coat, and silique elongation without fertilization. The method involved using a homozygous *Arabidopsis* line from Professor Daphne Preuss with a conditional male-sterile mutation (*pop1*). The *pop1* mutant pollen lacks the outer coating of the pollen. When homozygous *pop1* plants are grown at non-permissive conditions, low relative humidity, the mutant pollen fails to absorb (rehydrate) water from the stigma, which rejects the pollen with a callose barrier. At the permissive condition, high relative humidity, homozygous *pop1* plants make viable pollen, are hydrated on the stigma, and produce long siliques with many seeds. By contrast, homozygous *pop1* are sterile and display very small siliques with no viable seeds.

During my sabbatical, I worked in the greenhouse with a skilled undergraduate and we mutagenized homozygous *pop1* Arabidopsis seeds with ethyl methanesulfonate (EMS). We grew 50,000 M1 mutagenized *pop1* Arabidopsis plants at low-humidity conditions. The first line we identified displayed elongated siliques. However, we forgot to transfer it to high humidity to get viable seeds. So, the line was lost! But I told my undergraduate, and my wife, if it happened once, it would happen again. And it did. We identified 11 M1 plants that displayed elongated siliques. These lines were transferred to a growth chamber at high humidity (permissive conditions) and seed stocks were collected.

Although we did not detect embryo development, without fertilization we documented endosperm, maternal seed coat, and silique elongation. We initially named our eleven lines *fertilization-independent endosperm* (*fie-1 to fie-11*). I knew the *fie* mutations would become the basis of my research going forward, and gradually more members of my lab began to work on them. I put all my energy and effort into this project.

Morphological analysis showed that without fertilization the *fie* central cells proliferated, closely resembling wild-type fertilized endosperm development. The mutant *fie* central cell nucleus forms a syncytium, where the nuclei migrate from the micropylar end of the central cell and take up positions in the endosperm. Thus, the requirement for fertilization to initiate these early events in endosperm formation are eliminated by the *fie* mutations. The result suggested *fie* mutations influence a signal transduction pathway that prevents endosperm development prior to fertilization.

There are many mutations that result in embryo abortion, and they are usually recessive. Simple Mendelian genetics predicts that self-pollinated heterozygous plants will display 25% seed abortion in their siliques, a 1/4th chance that both the egg and sperm must carry the mutation. By contrast, self-pollinated heterozygous *FIE/fie* plants display 50% seed abortion in their siliques. How could Mendelian genetics be wrong? To address this question, we carried out reciprocal crosses between a heterozygote and wild-type plants. That is, female *FIE/fie* crossed with male wild-type results in a 50% seed abortion and 50% viable seeds. By contrast, the reciprocal cross, female wild type crossed with male *FIE/fie* results in progeny with no seed abortion. Therefore, inheritance of a maternal mutant *fie* allele resulted in embryo abortion, even when the paternal allele was wild type.

The 50% seed abortion phenotype described above is robust, and we used it for genetic mapping experiments. We first mapped the *fie* mutations with morphological markers and then more precisely with molecular markers. We then introduced overlapping wild-type DNA cosmid clones into heterozygous *fie-1* plants by Agrobacterium-mediated transformation. Cosmids that spanned the wild-type *FIE* gene complemented the *fie-1* mutation. Because the *fie-1* mutation is a female gametophytic lethal, not an embryo lethal, prior experiments did not reveal if the *fie* mutant allele was recessive or dominant. However, the complementation experiments indicated *fie-1* is recessive to wild-type, suggesting that maternal wild-type FIE allele represses endosperm development before fertilization.

I will never forget when my postdoctoral fellow, Ramin Yadegari, came into my office and said, “I have something I want you to see.” I went with him to the computer screen, and there it was, the *FIE* gene. This was over a year from mutagenesis to cloning the gene. Sequencing the wild-type gene and cDNA clones revealed the *FIE* gene structure. Each mutant gene had a loss-of-function mutation. FIE encodes a WD-repeat protein homologous to the WD-repeat Polycomb group (PcG) repressive complex 2 (PRC2). By contrast, we mapped the *fie-11* mutant allele to a gene that encodes a SET-domain PRC2

protein. This gene was independently discovered and named *MEDEA* (*MEA*) by Ueli Grossniklaus as a maternal allele required for embryogenesis. The WD-repeat Polycomb and the SET-domain Polycomb protein bind together in the PRC2 complex and were originally identified in insects and mammals where they function to repress gene transcription.

This research revealed the power of genetics to identify genes in pathways and complexes, as we and others pinpointed components of the Polycomb group (PcG) repressive complex 2 (PRC2). Using the yeast 2-hybrid system, we showed that the FIE and MEA Polycomb proteins interact physically, suggesting the molecular partnership of WD and SET domain Polycomb proteins has been conserved during the evolution of flowering plants.

Only maternal *MEDEA* mRNA is expressed in the *Arabidopsis* endosperm.

Inheritance of a maternal loss-of-function *mea* allele results in embryo abortion and prolonged endosperm production, even when the wild-type MEA paternal allele is inherited. Thus, only the maternal wild-type *MEA* allele is required for proper embryo and endosperm development. To understand the molecular mechanism responsible for the parent-of-origin effect of the *MEA* allele on seed development, we compared the expression of maternal and paternal wild-type *MEA* alleles in the progeny of reciprocal crosses between two Arabidopsis ecotypes. The ecotypes, separated for many years, accumulate sequence differences, in this case, a DNA restriction endonuclease site that differentiates mRNA converted to DNA. Only the maternal *MEA* mRNA was detected in endosperm from seeds at the embryo torpedo stage. In contrast, expression of both maternal and paternal *MEA* alleles was observed in the embryo from seeds at the torpedo stage, and later in seedling, leaf, stem, and root. *MEA* was the first authenticated gene to display parent-of-origin–dependent monoallelic expression specifically in the endosperm. The results suggest embryo abortion in mutant *mea* seeds is due, at least in part, to a defect in endosperm development. It also suggests that *MEA* parent-of-origin-dependent monoallelic expression is generated by epigenetic gene imprinting.

Why is parent-of-origin gene expression important?

Eutherian mammals with a placenta and angiosperm plants with a seed endosperm, exceedingly different biological kingdoms, have independently evolved innovations that support nourishment of their developing embryos and enhance their chances of successful reproduction. The placenta, composed of maternal and fetal tissues, facilitates nutrient and gas exchange between the mother and the developing fetus; the triploid endosperm tissue provides nutrition for the embryo. Sexual reproduction, involving meiosis to produce gametes for subsequent fertilization, is responsible for the vast diversity of angiosperms that evolved on earth.

Reproduction for mammals and plants creates a metabolic burden that is not evenly distributed between the female and male parents. Both parents contribute a haploid genome to the progeny, however, in both mammals and angiosperm plants, it is the female parent, with its placenta or endosperm, that nourishes the developing embryo. This disparity is called the parent-offspring conflict. How are the selective pressures on each parent resolved? When there are multiple male parents, each one competes to have the embryo receiving their genome acquire the most nourishment from the maternal parent; consequently, progeny containing their genome would have the best chance to survive. In contrast, the strategy of the female parent is to equally allocate nutrition to all the embryos, so that she has the best chance to successfully produce progeny containing her genome.

Our data indicate that the MEA-FIE Polycomb complex is one means by which the maternal genome modifies the activity of the paternal genome. It illustrates the prominent role of the maternal genome to control the paternal genome by endosperm imprinting during seed development. To understand the mechanism that regulates MEA parent-of-origin expression, we must introduce the *DEMETER* (*DME*) gene.

The DME maternal gene is essential for seed viability.

My lab technician, Mike Hannon, came into my office and asked that instead of discarding 5,000 T-DNA mutagenized plants, why don't we look for rare lines with 50% seed abortion. Maybe we might find a new Polycomb gene. I give him all the credit for asking the question. What Mike discovered was a new gene, one with a novel mechanism that regulates Polycomb genes and more.

We named the gene *DEMETER* (*DME* for demethylation), the Greek goddess of fertility, harvest and agriculture.

Led by postdoctoral fellow Yeonhee Choi, we verified that self-pollinated *DME/dme* generated 50% seed abortion. As we did with heterozygous *MEA/mea* and *FIE/fie* plants, we did reciprocal crosses with *DME/dme* and wildtype. When *DME/dme* was the maternal parent, the progeny generated 50% aborted seeds and the remaining 50% viable seeds were wildtype. When *DME/dme* was the male parent, the progeny generated 100% viable seeds, 50% were heterozygous *DME/dme* and 50% were wildtype. Thus, seed viability depends only on the presence of a wild-type maternal *DME* allele, as do the maternal *MEA* and *FIE* alleles, and the paternal alleles are expendable. The *dme-2* and *dme-3* alleles are loss-of-function mutants, as their respective T-DNAs were inserted into the middle portion of the *DME* gene.

DME is related to DNA glycosylase/lyase base-excision DNA repair enzymes.

We obtained full-length *DME* cDNA clones that predicted the amino acid sequence of the DME protein. A conserved domain search of NCBI databases revealed domains related to the helix-hairpin-helix superfamily of base excision DNA repair proteins. All organisms have families of base-excision DNA repair enzymes. Simply said, they repair DNA by excising a damaged or mismatched base, followed by a DNA polymerase that inserts the correct base. However, if DME is an active base excision repair enzyme, what base does it excise?

Collaborating with Roger Pennell at Ceres, Inc., we turned to genetics to get a clue about DME's substrate by mutagenizing *DME/dme* to create a suppressor gene that rescues seeds with a maternal *dme* allele. We opened siliques of 8,000 mutagenized plants and looked for the rare plant with only 25% seed abortion. That is, 25% inherit maternal *dme* and the seeds abort, 25% viable seeds inherit maternal *dme* allele and suppressor allele, 25% viable seeds inherit maternal wild-type *DME* and suppressor, and 25% viable seeds inherit maternal wild-type *DME*. We found four suppressor lines each with an independent loss-of-function mutation in the *MET1* gene. The MET1 enzyme methylates cytosine to 5-methylcytosine bases in DNA in the CG sequence context. This suggests that *DME* and *MET1* have antagonistic functions, MET1 is a DNA methylation enzyme and DME is a DNA demethylation enzyme.

***DME* is primarily expressed in the central cell**

To visualize *DME* gene expression, we transformed *Arabidopsis* plants with a chimeric gene, a DME promoter fused to the *GREEN FLUORESCENT PROTEIN* (*DME::GFP*) reporter gene. In the mature unfertilized female gametophyte, we detected GFP fluorescence only in the central cell. After fertilization, *DME::GFP* promoter activity rapidly decreased. GFP fluorescence was no longer detected prior to the first division of the primary endosperm nucleus, nor in endosperm or embryo development. These results show *DME* promoter activity and DME protein function in the female gametophyte central cell before fertilization.

***DME* expression in the central cell is necessary for expression of *MEA* and *FIE* in the central cell and endosperm.**

To understand the regulation of *MEA* gene expression by *DME* during seed development, we observed the effect of *dme* mutation on transcription of a *MEA::GFP* transgene. As predicted, 50% of pre-fertilization ovules from transgenic plants hemizygous for the *MEA::GFP* transgene displayed strong fluorescence in the central cell nucleus and cytoplasm prior to fertilization. In a plant hemizygous for the *MEA::GFP* transgene and heterozygous *DME /dme*, 25% of the pre-fertilization ovules displayed GFP fluorescence in their central cells, suggesting a wild-type *DME* allele is necessary for transcription of the *MEA::GFP* transgene in the central cell of the female gametophyte prior to fertilization.

When flowers hemizygous for the *MEA::GFP* transgene and heterozygous *DME/dme* were pollinated with wild-type non-transgenic pollen, we observed approximately 25% seeds with GFP fluorescence in endosperm cells after pollination. This result suggests a wild-type DME, expressed only in the pre-fertilization central cell, is necessary for sustained expression of the *MEA::GFP* transgene in the endosperm after fertilization. Thus, parent-of-origin effects of *dme* mutations on seed viability are due, at least in part, to a failure to express the maternal *MEA* allele in the central cell during female gametophyte development.

DME initiates an active DNA demethylation pathway.

The enzymes and mechanisms that methylate cytosine are well known. When we started this research there was no known mechanism, enzyme, or enzymatic path for active enzymatic DNA demethylation. Laboratories searched for an enzyme that would directly remove the 5-methyl group from cytosine for years, but without success. This is likely due to the exceedingly stable carbon-carbon bond of the 5-methyl group. We showed that the DME DNA glycosylase initiates an active DNA demethylation pathway in plants.

DNA glycosylase/lyases represent a diverse array of small (200–300 amino acids), monomeric, structurally related DNA repair proteins that are highly conserved in evolution. These proteins excise mismatched or altered bases (e.g., oxidized, deaminated, alkylated, and methylated) in the DNA. Although DME is a much larger protein, it has all the essential conserved sequences in other DNA glycosylase/lyases. Our hypothesis was that DME flips out a methylated cytosine (5-methylcytosine) from the double helix and uses its glycosylase activity to cleave the covalent bond, releasing 5-methylcytosine from the deoxyribose sugar and concomitantly cleaving the DNA phosphodiester bond. DME's job is done, leaving an empty site (no base) in the DNA. Downstream enzymes service all the DNA glycosylase/lyases. An AP (no purine/no pyrimidine) endonuclease generates a 3'-hydroxyl that is used by a DNA repair polymerase, which in DME's case inserts a single cytosine deoxynucleotide triphosphate (note that there is no 5-methylcytosine deoxynucleotide triphosphates). Then a DNA ligase seals the nick in the DNA.

To test our hypothesis biochemically, we expressed DME in *E. coli* and purified the enzyme. We incubated DME with P^{32}-end-labeled double-strand oligonucleotides with a single 5-methylcytosine (hemi-methylated substrate). DME broke the phosphodiester linkage at the 5-methylcytosine residue, and we could visualize all the predicted products on denaturing polyacrylamide gels. This verified DME functions as a DNA glycosylase/lyase. The only difference is its unique substrate, 5-methylcytosine.

DEMETER glycosylase/lyase establishes gene imprinting in the endosperm.

These data show how the DME DNA demethylation is the primary mechanism for maternal specific expression of the *FWA* homeodomain transcription gene (also named *FIS2* by Abed Chaudhury) in Arabidopsis endosperm. Next, we showed that the wild-type *FWA* transcription factor gene displays imprinted (maternal origin–specific) expression in the endosperm. The silencing *FWA* imprint depends on the maintenance a DNA methyltransferase, MET1, as is the case in mammals. Unlike mammals, however, the *FWA* imprint is not established by allele-specific *de novo* methylation. Rather, it is established by maternal gametophyte–specific gene activation, which depends on DNA demethylation by the DME DNA glycosylase gene. The paternal *FWA* allele is silent because its DNA is not demethylated. These experiments were a collaboration between my lab and Professors Tetsuji Kakutani and Steve Jacobsen.

This research shows that regulation of maternal *MEA* expression and paternal *MEA* silencing is regulated by two different mechanisms. Initially, prior to central cell formation and prior to the paternal MEA allele expression in the sperm, the MET1 DNA methyltransferase methylates regions upstream and downstream and there is no maternal or paternal MEA expression. When the central cell is mature, DME demethylates the regions upstream and downstream of the *MEA* gene, allowing the maternal allele to be expressed and the MEA-FIE Polycomb complex is assembled. However, the MEA-FIE Polycomb complex does not bind to the maternal *MEA* allele. Upon fertilization, the sperm enters the central cell and the MEA-FIE Polycomb complexes target and silence the paternal *MEA* allele. Unexpectantly, paternal-allele silencing is not controlled by DNA methylation. Rather, Polycomb group proteins that are expressed from the maternal genome, including MEA, control paternal MEA silencing. Thus, DME establishes MEA imprinting by removing 5-methylcytosine to activate the maternal allele. MEA imprinting is subsequently maintained in the endosperm by maternal MEA silencing the paternal allele.

Genomic DME DNA demethylation

This research was a collaboration with Daniel Zilberman at UC Berkeley. He taught me that studying the genome revealed correlations you can miss by studying only a single gene in depth. Cytosine methylation regulates gene expression and represses transposable elements (TEs) in plants and vertebrates. A novel method of genome sequencing was made possible by Illumina next-generation DNA sequencing technology. With

Illumina, we could sequence the Arabidopsis genome in a matter of days. By comparing this sequence to that of bisulfite treated DNA, we could determine the site of every 5-methylcytosine and its prevalence in the Arabidopsis genome.

The DME DNA glycosylase that excises 5-methylcytosine is highly expressed in the central cell before fertilization and is required for the demethylation observed in endosperm, which has been inferred to occur on the maternal chromosomes inherited from the central cell. However, DNA methylation of the maternal and paternal endosperm genomes had not been compared except for a few loci, and therefore it is difficult to make general inferences about the mechanism and specificity of central cell demethylation.

To understand the extent, mechanism, and biological significance of active demethylation in the central cell, we used bisulfite sequencing of reciprocal crosses between the Col and Ler ecotypes of Arabidopsis that differ by 400,000 single-nucleotide polymorphisms (SNPs) to identify DNA methylation that resides on either the maternal or paternal endosperm genome. The wild-type maternal genome displays strong localized demethylation compared to the paternal genome. The demethylation pattern is near fully reversed in *dme* mutant endosperm, which indicates that DME is the major enzyme required for excision of 5-methylcytosine in the central cell and demonstrating that active DNA demethylation of at least 10,000 specific sequences spanning 4,500,000 base pairs accounts for the methylation differences between the maternal and paternal endosperm.

DME-mediated DNA demethylation in the central cell is required to establish monoallelic (imprinted) expression of genes in the endosperm. We examined the location of loci that are significantly less methylated in wildtype endosperm than in *dme* endosperm in relation to imprinted endosperm genes. Maternally and paternally expressed genes are preferentially associated with differentially methylated regions, particularly just upstream of the gene. Maternally expressed genes also exhibit differentially methylated regions that span the transcriptional start site, consistent with the strong correlation between methylation of this region and gene silencing. These regions are in positions that regulate expression of genes and transposons.

DME DNA demethylation in Arabidopsis and rice central cells.

For this research my collaborators were Professors Yeonhee Choi at Seoul National University, Daniel Zilberman at UC Berkeley and Takashi Okamoto at Tokyo Metropolitan University. DME demethylates DNA and is expressed in Arabidopsis central cells. The maternal endosperm chromosomes inherited from the central cell are also extensively demethylated at similar sequences in Arabidopsis. Demethylation of maternal endosperm chromosomes requires DME in Arabidopsis, and loss of DME function disrupts endosperm gene expression, gene imprinting, and causes seeds to abort. Several lines of evidence strongly argue that the demethylation observed in the endosperm is inherited from the central cell: Only the central cell-derived chromosomes are demethylated, DME is rapidly down-regulated following sperm fusion, and genes activated by demethylation are expressed in the central cell. However, DNA methylation had not been analyzed in the Arabidopsis central cell, leaving the origin of endosperm demethylation uncertain.

We used a novel technique, INTACT (Isolation of Nuclei Tagged in specific Cell Types), to isolate central cell nuclei from wild-type Arabidopsis plants. When the analysis is confined to sequences that show demethylation of maternal chromosomes in the endosperm, almost all of the loci are hypomethylated in the central cell. To determine whether central cell demethylation requires DME, we isolated central cells from heterozygous *DME/dme* plants. Although the overall patterns of CG methylation are similar between central cells from wild-type and *DME/dme* plants, loci demethylated on maternal endosperm chromosomes are more extensively methylated in central cells of *DME/dme* plants, as they are in *dme* endosperm. The methylation levels at these loci in central cells from *DME/dme-2* plants are about halfway between those of wild-type central cells and those of loci that are not demethylated in endosperm, as would be expected if only half of the central cells harbor the *dme-2* mutation. Taken together, these results demonstrate that the DME-dependent demethylation observed on maternal endosperm chromosomes is initiated in the central cell. Following my description of these results, my colleague,

Professor Anna Koltunow, sent me an email saying, "You finally proved that DME demethylates the central cell! All this time it was only a hypothesis. Congratulations!"

To complement our Arabidopsis experiments, we analyzed DNA methylation in central and egg cells of rice. We isolated rice central cells and egg cells by microdissection (and obtained whole-genome DNA methylation data for both cell types). Central cell CG methylation levels are lower than those of embryos, roots, and leaves, resembling most closely the methylation of endosperm, whereas CG methylation in the egg cell is slightly elevated compared with other cells. Taken together, these data indicate that the DNA demethylation of maternal endosperm chromosomes observed in Arabidopsis and rice plants is initiated in the central cell.

Rice and Arabidopsis shared a common ancestor about 150 million years ago, as is true of humans and kangaroos. Plant genome sequencing invariably reveals DME-related genes, which suggests DNA demethylation in the central cell is highly conserved among plants.

Summary

My fascination with the function of the female gametophyte and the central cell began with finding Arabidopsis mutations that revealed aspects of apomixis – fertilization independent endosperm, seed coat development, and silique elongation. The genes we discovered, *FIE* and *MEA*, encode a Polycomb complex expressed in the central cell that suppresses these developmental processes until fertilization. Only the maternal *FIE* and *MEA* alleles are necessary for seed viability, which breaks Mendel's laws. DME, a DNA demethylation enzyme expressed only in the central cell, activates expression of FIE and MEA that continue to function in the endosperm. Without DME, *FIE* and *MEA* maternal expression, the seed will abort. DME and Polycomb genes regulate parent-of-origin gene imprinting. By studying DNA demethylation in entire genomes, we proved DNA demethylation in the central cell is a conserved process necessary for producing the endosperm that nourishes the embryo. It also functions in an uncountable number of organisms on this planet.

In concluding, I would like to describe how I feel about the importance of teaching at the university. I taught a molecular biology and genetics class to over 500 students each year. It was an uplifting part of my job. I carefully stacked concepts, so that Monday's new information could be used for Wednesday's new information. I didn't try to cover everything in the textbook. Rather, I gathered the most important concepts that students needed to build their knowledge in subsequent classes. Going deeper into a topic makes it possible to use new information. It is important to create a unified picture of what you are teaching. Treasure the questions students ask, as this dialog is shared with the entire class. They paid the most attention when they didn't know how I would answer the question. And one question can lead to another, and to another, and learning happens. I always praised the questioner.

What advice would you offer a young person considering a career in plant biology.

Learn as much as you can. Having a broad, deep knowledge base gives you options for your career. Seek out your teachers and senior students in classes. They can be mentors and have an impact on your career. It is good to work in groups, but in the end you must understand biological concepts in your own mind. If you don't understand, there is a missing link in your reasoning. Get help; ask questions. If you don't ask, you may never understand. If you ask, you will reveal some lack of knowledge, but you will remember the answer forever.

Whenever I learned something new, I wrote it down. For many people, the physical act of writing, drawing pictures, and/or making tables, imprints knowledge deeper into your memory, where it's usable when needed. If you are working in a plant biology laboratory, be sure you have a mentor(s). This will help you to clearly understand the goal of an experiment, how the experiment is done, what results you expect, and whether you can use them to make an hypothesis for the next experiment.

Think about what interests you in plant biology. There are many career opportunities. Would you like to teach at a university, as a professor or technician, or teach at a high school, city college, a state college? Would you prefer to work at a small start-up company or a large company that produces better seeds for first-, second- or third world nations? Or perhaps you prefer to work as a lawyer who protects farmers or consumers that receive agricultural

products at a reasonable price? Or do you want to go to the Peace Corps and teach better farming practices to third-world farmers? Talk to people in the different fields and find out what fits your personality, ideals, and mentality. Be courageous if you change your field.

A career can be lifelong if you enjoy it. It is a marathon, not a sprint. Some people find success very early, and some (like me) find it later in their career. Never give up, keep trying, and you will be content with your decisions and career.

People who greatly contributed to research in my laboratory are listed below in alphabetical order:

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